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Gastrin increases its own synthesis in gastrointestinal cancer cells via the CCK2 receptor

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ABSTRACT

The involvement of the gastrointestinal hormone gastrin in the development of gastrointestinal cancer is highly controversial. Here we demonstrate a positive-feedback loop whereby gastrin, acting via the CCK2 receptor, increases its own expression. Such an autocrine loop has not previously been reported for any other gastrointestinal hormone. Gastrin promoter activation was dependent on the MAP kinase pathway and did not involve Sp1 binding sites or epidermal growth factor receptor transactivation. As the treatment of gastrointestinal cancer cells with amidated gastrin led to increased expression of non-amidated gastrins, the positive-feedback loop may contribute to the sustained increase in circulating gastrins observed in colorectal cancer patients.

1. Introduction

Amidated gastrin (Gamide) is responsible for gastric acid secretion as well as for maintenance of the integrity of the gastric mucosa. Gastrin gene expression is regulated by acid via a negative-feedback loop. In response to gastric antral distension, pH elevation or contact with amino acids and peptides during a meal, gastrin is released from antral G cells into the circulation [1]. Gastrin then stimulates the release of gastric acid, causing gastric pH to fall. Low pH in turn activates the release of somatostatin, the inhibitor of gastrin release, and circulating gastrin returns to basal levels [2].

Gamide is processed from an 80 amino acid precursor (progastrin) by a series of enzymatic cleavages and by transamidation [1]. There is now abundant evidence that non-amidated gastrins like progastrin and glycine-extended gastrin (Ggly) act as growth factors for the normal colorectal mucosa, and stimulate the development of colorectal carcinoma in animals [3–5]. A connection between Gamide and some forms of gastric cancer is firmly established in animal models but less clearcut in humans (reviewed by [6,7]). All the actions of amidated gastrins are mediated via the cholecystokinin-2 receptor (CCK2R), but the identity of the receptor(s) for non-amidated gastrins remains unknown.

In this paper we have investigated by quantitative PCR and by luciferase reporter assay whether or not gastrin can regulate its own expression in gastrointestinal cancer cell lines. Herein we report the unexpected discovery that gastrin activates its own transcription in a positive-feedback loop via the CCK2R. The resultant increased production of non-amidated forms of gastrin (Ggly) may explain the sustained increase in circulating gastrins in patients with colorectal cancer (CRC).

2. Materials and methods

2.1. Cell lines

The following human cell lines were used: AGS wildtype (WT), AGS transfected with CCK2R cDNA (AGS CCK2R), Colo320 WT, Colo320 transfected with CCK2R cDNA (Colo320 CCK2R), Lovo and JURKAT. All cells were maintained in RPMI supplemented with 8% FBS and grown in a humidified incubator at 37 °C.

2.2. Amidated and non-amidated gastrin radioimmunoassay

Region-specific gastrin antisera were used to measure amidated gastrin (antiserum 1296) and its precursor glycine-extended gastrin (antiserum 7270) by radioimmunoassay as described previously [8]. The crossreactivity of antiserum 7270 for amidated gastrin is 0.07.

Abbreviations: CCK2R, cholecystokinin-2 receptor; EGFR, epidermal growth factor receptor; CRC, colorectal cancer; Luc, luciferase; MAPK, mitogen activated protein kinase

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2.3. Plasmid constructs

The basic luciferase vector pFR-luciferase (Luc) containing 1300 bp of the human gastrin promoter region and the first exon of the gastrin gene (1300pFR-GASLuc) was a generous gift from Professor J. Merchant (University of Michigan, Ann Arbor). The 1300 bp fragment was cloned into pGL3 basic vector and served as a template for preparation of five subsequent 5′-deleted gastrin promoter constructs, retaining 1147, 948, 666, 365 or 109 bp of the promoter region as well as the first exon. Restriction sites for *Nhe* I and *Bgl* II were introduced at the 5′ and 3′ ends, respectively, of each PCR product by polymerase chain reaction with the primers listed in Table 1. The fragments were ligated into the *Nhe* I and *Bgl* II sites of the pGL3 vector using T4 DNA ligase (Roche Diagnostics, Castle Hill, Australia). Site-directed mutagenesis of GC boxes 1 (GGCGGG → ACTAGT) and 2 (GGCGGG → GAATTC) within the 365 bp gastrin promoter construct was performed by introduction of unique restriction enzyme sites, SpeI and EcoRI, respectively. All constructs were verified by sequencing (Micromon, Monash University, Clayton, Australia).

2.4. Transfection

Lipofectamine LTX or Lipofectamine 2000 (Invitrogen, Melbourne, Australia) was used for transfection according to the manufacturer's instructions. Briefly, cells were seeded at a density of $0.5–1.0 \times 10^5$/well in 24-well plates 16 h prior to transfection. Cells had usually reached 70–80% confluency at the time of transfection and were washed once with Opti-MEM prior to the procedure. Each well was transfected with 0.5–1 μg of the test plasmid in 500 μl Opti-MEM containing 2 μl of lipofectamine reagent. After 6 h the medium was replaced with fresh RPMI medium supplemented with 8% FBS (1ml/well) and cells were incubated at 37 °C for a further 24-48 h before assay.

2.5. Reporter gene assay

Luciferase activity was determined using the Luciferase Assay kit (Promega, Hawthorn, Australia). Briefly, cells were lysed using 60–70 μl/well reporter lysis buffer on a shaker at room temperature for 20 min. Aliquots of 15–20 μl lysate from each sample were distributed into wells on opaque 96-well microtitre plates and an equal amount of LAR-II (firefly substrate) was added. Luciferase activity was measured with a MicroLumi XS luminometer (Harta Instruments, Gaithersburg, MD, USA). Luciferase activity was normalised to the total protein content of each well determined using the Bradford reagent (Bio-Rad, Gladesville, Australia).

Table 1
List of primers used to produce the GASLuc constructs.

Primers	Sequence (5′–3′)
p1,147-GASLuc forward	GGGGTACCCTGTTTGCTAACCATTTGAG
p948-GASLuc forward	GGGGTACCGCGCCTGTAATCCCAGCTC
p666-GASLuc forward	GGGGTACCGGGCAGCTACAAGCCAAAC
p365-GASLuc forward	GGGGTACCACCTACCACCACCCACAAG
GASLuc reverse – for all gastrin promoter constructs	GAAGATCTCTGCAGAGCTGGGAGGTG
GC Box2 mutagenesis	
Forward	TGGAATTCGCAGGGTGATGGGCTG
Reverse	GCGAATTCCTTTCATTTATGTTCAGTCAGTCATCC
GC Box1 mutagenesis	
Forward	GGACTAGTGTGGGGGGACAGTTGGG
Reverse	GTACTAGTCCTACCCTGCCATATGAGTCC

2.6. RNA preparation and quantitative PCR of gastrin mRNA

Cells were seeded at a density of 2.5×10^5/well in 6-well plates in growth media. Next day cells were treated and the following day total RNA was isolated from cells using TRIzol reagent (Invitrogen, Melbourne, Australia) according to the manufacturer's instructions. Five micrograms of total RNA was used for cDNA synthesis with the Superscript™ II first strand synthesis system (Invitrogen). The resulting cDNA transcripts of mRNA were used for real time PCR amplification with the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Melbourne, Australia) and Taqman chemistry. The following primers were used: forward 5′-CCGCAGTGCTGAGGATGAG-3′, reverse 5′-GGAGGTGGCTAGGCTCTGAA-3′, probe 5′-CTAACAATCCTAGAACCAAG-3′. Gene expression was quantitated relative to 18S RNA expression.

2.7. Statistical analysis

Statistical significance for single comparisons of normally distributed data was assessed by Student's *t*-test or for data that was not normally distributed by Mann–Whitney rank sum test. For multiple comparisons one-way ANOVA was used. All statistics were analysed with the statistical program SigmaStat and graphed using SigmaPlot (Jandel Scientific, San Rafael, CA).

3. Results

3.1. Gastrin activates its own transcription via the CCK2R

To determine whether or not gastrin upregulates its own expression, gastrin mRNA was measured in a number of gastrointestinal cancer cell lines with and without treatment with Gamide. In AGS CCK2R cells, gastrin gene transcription was activated by 31- and 44-fold with 10 and 50 nM Gamide, respectively. Significant but smaller increases were observed in Colo320 CCK2R cells (1.5- and 4.4-fold with 10 and 50 nM Gamide, respectively) and in JURKAT cells that express endogenous CCK2R (2.5- and 3.6-fold with 10 and 50 nM Gamide, respectively). Gastrin gene transcription was activated only in those cells that expressed the CCK2R, whether the receptor was endogenously expressed (JURKAT) or transfected (AGS CCK2R and Colo320 CCK2R) (Fig. 1A). When Ggly was measured as a marker of an increase in translation of the gastrin gene, cell lines that expressed the CCK2R also released more Ggly into the cell growth media following stimulation with Gamide (Fig. 1B). Although the presence of exogenous Gamide prevented the measurement of endogenous Gamide in conditioned media, the detection of secreted Gamide in the media conditioned by untreated AGS or AGS CCK2R cells (1.7 ± 0.9 and 3.1 ± 1.4 fmol/ml, respectively) clearly established that the peptidyl α-amidating monooxygenase was present and capable of converting Ggly into Gamide. The AGS CCK2R cell line was chosen for further studies because it showed the greatest increase in gastrin mRNA synthesis.

3.2. Activation of the gastrin promoter is time-dependent

The dose response and time course of gastrin transcription were next examined. Promoter activation was measured in a luciferase reporter assay with AGS CCK2R cells transfected with a pGL3 luciferase reporter vector containing 1300 bp of the human gastrin promoter. Promoter activity increased following the addition of 10, 50 or 100 nM of Gamide (Fig. 2A), and a concentration of 50 nM was chosen for subsequent experiments. Time course experiments indicated that promoter activation was maximal at 8 h (Fig. 2B). Measurement of gastrin mRNA by quantitative RT-PCR indicated that transcription continued to increase up to at least 16 h (Fig. 2C).

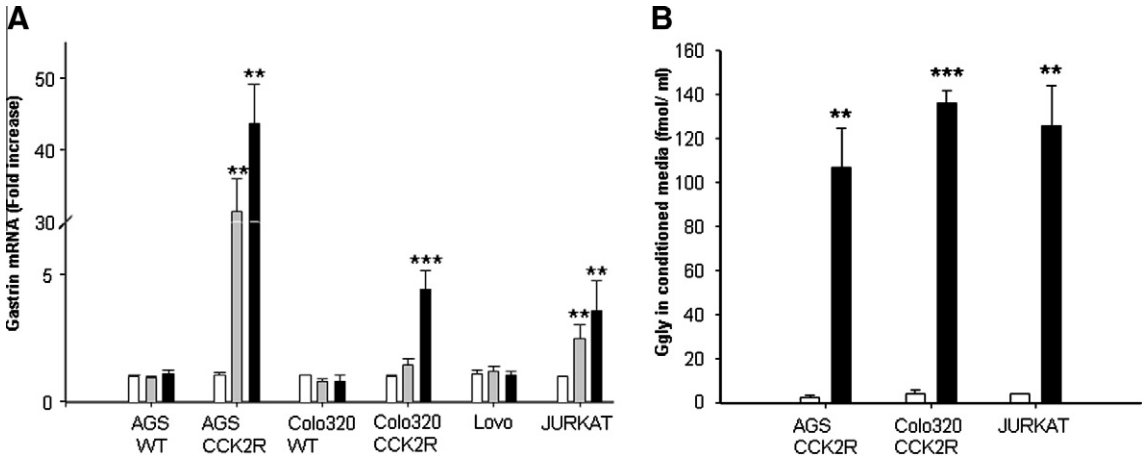

Fig. 1. Gastrin increases gastrin mRNA transcription via the CCK2 receptor. (A) A number of gastrointestinal cell lines with or without CCK2 receptors were stimulated with 10 nM (grey bars) or 50 nM (black bars) Gamide for 16 h. Gastrin mRNA was subsequently measured using real time PCR and expressed relative to the amount of 18S rRNA. The ratio was compared to the value for unstimulated cells (white bars). (B) Increased activation of gastrin mRNA transcription in AGS CCK2R, Colo320 CCK2R and JURKAT cells by 50 nM Gamide led to increased Ggly concentrations in the conditioned medium. Data are mean ± S.E.M. ($n = 3$; $^{**}P < 0.01$; $^{***}P < 0.001$).

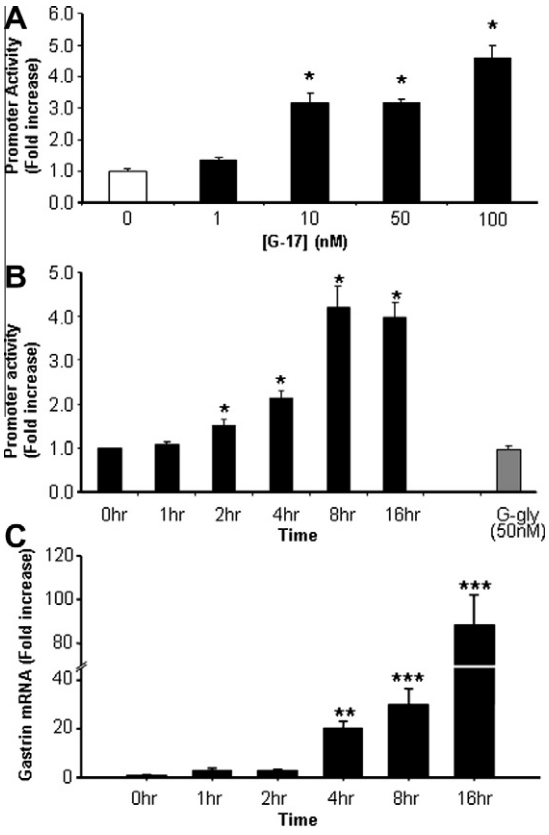

Fig. 2. AGS CCK2R cells stimulated with 50 nM Gamide increase gastrin mRNA and promoter activity in a time-dependent manner. (A) The gastrin promoter is activated in a dose-dependent manner. Cells were treated with 1, 10, 50 and 100 nM of Gamide for 16 h. A concentration of 50 nM was chosen for subsequent experiments. (B) Cells were treated with 50 nM Gamide for the indicated times, and activation of the gastrin promoter was measured using luciferase reporter assay. Ggly did not stimulate promoter activation. (C) Cells were treated with 50 nM Gamide for the indicated times, and gastrin mRNA was subsequently measured using real time PCR and expressed relative to the amount of 18S rRNA. The ratio was compared to the value for unstimulated cells. The time course for activation of transcription of gastrin mRNA matched closely with the time course for promoter activation. Data are mean ± S.E.M. ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Stimulation with 50 nM Ggly did not increase promoter activity (Fig. 2B) or gastrin mRNA (data not shown).

3.3. A minimal human gastrin promoter is responsible for the activation of the gastrin gene

To identify the regions of the human gastrin promoter required for activation by Gamide, sequential deletion mutants retaining from 1147 to 365 bp of the human gastrin promoter were constructed using PCR techniques. The mutants were transfected into AGS CCK2R cells and their basal activities assayed. All deletion constructs of the gastrin promoter (1147, 948, 666 and 365 bp) showed similar increases in activity in response to Gamide treatment (Fig. 3).

3.4. Gastrin activates the gastrin positive-feedback loop via the MAP kinase pathway

To determine which signalling pathways downstream of the CCK2R were involved in the Gamide-dependent activation of the human gastrin promoter a panel of receptor antagonists and protein kinase inhibitors was tested. The CCK2R antagonist (RP73870A) completely blocked Gamide-dependent promoter activity. In addition, the specific mitogen activated protein kinase (MAPK) inhibitor (U0126) reduced Gamide-dependent promoter activation to control levels, while the inhibitor Sorafenib (which targets a tyrosine kinase upstream of the MAPK pathway [9]) also inhibited gastrin promoter activity to a similar extent. Interestingly, the PI3 kinase inhibitor (Ly294002) did not inhibit Gamide-dependent promoter activation (Fig. 4). The failure of T-25 (an inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase) to abrogate Gamide-dependent promoter activation suggests that the EGFR is not involved in the enhancement of gastrin transcription (Fig. 4).

3.5. Activation of the gastrin promoter by gastrin is Sp1-independent

To dissect further the mechanism of transcriptional activation of the gastrin gene, known transcription factor binding sites within the shortest active promoter construct (p365-GASLuc) were mutated by site-directed mutagenesis. The observations that activation of the CCK2R leads to activation of the MAPK pathway [10–12], and that MAPK can phosphorylate Sp1 [13], suggested that the Sp1 elements within the human gastrin promoter (Fig. 5A) were likely candidates. However, upon stimulation with Gamide no differences were observed in the luciferase activity between the mutated and wild-type gastrin promoter constructs (Fig. 5B).

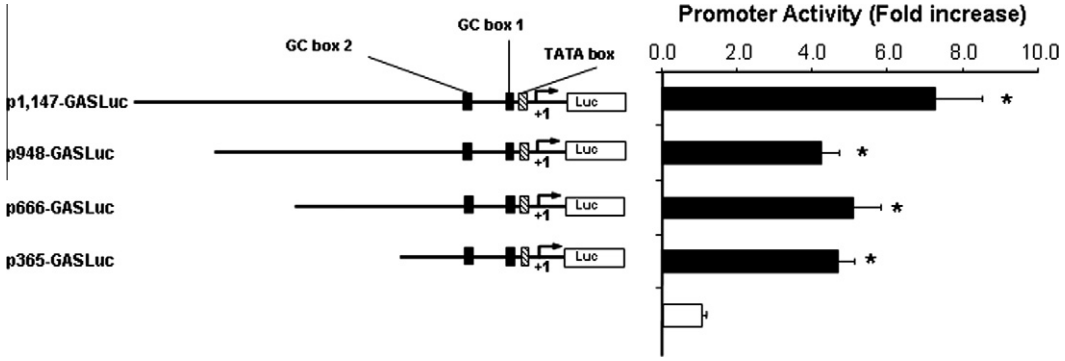

Fig. 3. Comparison of promoter activities following Gamide treatment in AGS CCK2R cells. (A) Gastrin promoter-reporter constructs with 5′ deletions were constructed as described in the Section 2. The promoter deletion constructs were individually transfected into AGS CCK2R cells. Luciferase activity of each vector was measured, normalised to the total protein concentration, and the value expressed as a percentage of that observed for p1147-GASLuc without Gamide treatment (white bar). Promoter activity of all constructs was increased following Gamide treatment (black bars). Data are mean ± S.E.M. ($n = 3$, $^{*}P < 0.05$).

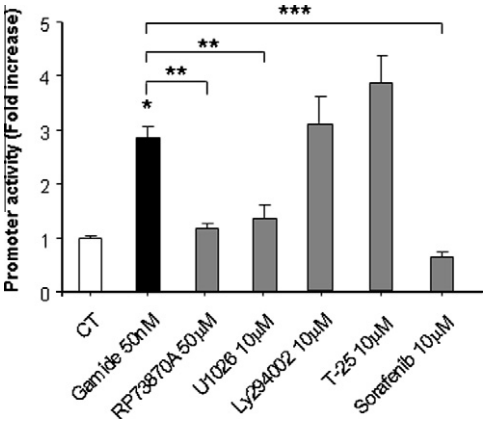

Fig. 4. Gastrin activates the gastrin positive-feedback loop via the MAPK pathway. AGS CCK2R cells transfected with the shortest active fragment of the human gastrin promoter, p365-GASLuc, were treated with a panel of receptor antagonist and kinase inhibitors (grey bars) 30 min prior to the addition of 50 nM Gamide (black bar). Gamide stimulation is calculated compared to unstimulated cells (white bar). The CCK2R antagonist RP73870A, the MAPK inhibitor U0126 and the MAPK-specific tyrosine kinase inhibitor, Sorafenib, all inhibited activation of the human gastrin promoter in the luciferase reporter assay. The PI3K inhibitor Ly294002 and the EGFR inhibitor T-25 had no effect on gastrin promoter activity. Data are mean ± S.E.M. ($n = 3$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

In addition assays with a further deletion construct p109-GASLuc which contained only the proximal Sp1 site showed no reduction in promoter activity (data not shown). Taken together the results indicate that activation of the gastrin promoter by Gamide is Sp1-independent.

4. Discussion

Although gastrin was discovered over 100 years ago its direct involvement in the growth of gastrointestinal cell lines and tumours remains controversial. Here we demonstrate by quantitative PCR and by luciferase reporter assay that Gamide is able to activate its own promoter, and hence transcription of the gastrin gene, in a time-dependent manner. To our knowledge such a positive-feedback loop has not yet been linked to the mechanism of any other gastrointestinal hormone, although interleukin-1 has been reported to induce its own expression via the interleukin-1 receptor in human mesangial cells [14]. A more usual positive-feedback loop consists of one growth factor activating the synthesis of another thus potentiating the growth signal [15]. The observation that Gamide increases its own transcription is also in contrast to the more common ligand-dependent repression by which gene products prevent their own over-expression [16].

The increase in transcription of the gastrin gene also results in increased translation. Following stimulation with Gamide cell lines that expressed the CCK2R also released more Ggly into the cell growth media (Fig. 1B). Unfortunately the addition of excess Gamide into the media at concentrations far exceeding the amount of Gamide produced endogenously made it impossible to measure the relatively small increases in endogenous Gamide. However, the fact that Gamide is produced by untreated AGS wild type and AGS CCK2R cells clearly established that the peptidyl α-amidating monooxygenase responsible for conversion of Ggly to Gamide is present and active in this cell line. Thus we conclude that an increase in Gamide production is also likely in response to Gamide treatment.

The positive-feedback loop for Gamide is dependent on expression of the CCK2R. Thus cells not expressing the CCK2R did not respond while in responsive cells a CCK2R antagonist completely blocked the Gamide-dependent increase in luciferase reporter activity. We were unable to find gastrointestinal cell lines expressing endogenous CCK2 receptors to confirm our findings in a non-transfected cell line, as even Lovo cells, which have been reported to express endogenous CCK2R [17], failed to bind gastrin in our hands (data not shown). However, the presence of the gastrin positive-feedback loop was detected in a lymphoblastoid T cell line (JURKAT) which is known to express endogenous CCK2R [18]. Interestingly, Gamide has also been shown to upregulate the expression of the CCK2R [19] thereby potentiating the positive-feedback loop.

The signalling pathways involved were investigated with specific inhibitors. One of the major signalling pathways activated downstream of the CCK2R is the MAPK cascade [10–12]. The observation that two inhibitors of the MAPK pathway completely blocked the Gamide-dependent increase in luciferase reporter activity indicates that MAPK is indeed the major pathway activated by Gamide via the CCK2R in the positive-feedback loop. In addition, Gamide transactivates the EGFR by inducing the expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF) which binds to EGFR, causing tyrosine phosphorylation of the EGFR and subsequent activation of downstream signalling pathways [11,20,21]. However, the observation that an EGFR inhibitor did not prevent the Gamide-dependent promoter activation is consistent with a direct effect of Gamide on the activation of its own promoter.

Gamide stimulates the expression of genes required for acid secretion and for cell proliferation and migration. For example

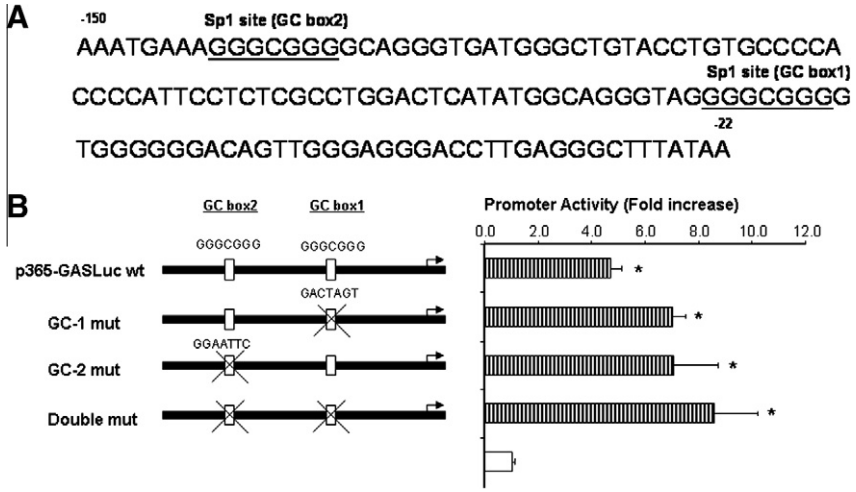

Fig. 5. Activation of the gastrin promoter by gastrin is Sp1-independent. (A) Sequence of the gastrin promoter demonstrating the location of two Sp1 sites within deletion construct p365-GASLuc. (B) Effect of site-directed mutagenesis of the Sp1 sites in p365-GASLuc showing that the gastrin positive feedback loop is not mediated via the Sp1 sites. Data are mean ± S.E.M. ($n = 3$, $^*P < 0.05$).

Gamide, acting via gastrin-response elements (GRE), activates the genes encoding vesicular monoamine transporter type 2 [22] and histidine decarboxylase [23]. Although no GREs were identified within the gastrin gene, the promoter does contain a number of Sp1 sites which are involved in activation of gastrin expression by EGF [24]. Using sequential deletion fragments of the human gastrin promoter, responsiveness to exogenous Gamide was shown to lie within the proximal 365 bp of the promoter. Although this region contains two Sp1 sites, the activation of the gastrin promoter by exogenous Gamide does not appear to be mediated via either site, since mutation of either or both Sp1 sites did not affect Gamide responsiveness in the luciferase reporter assay.

One potentially important functional consequence of increased gastrin gene transcription is an increased secretion of the immature forms of gastrin (Ggly) into the cell culture medium (Fig. 1B). Hence in CRC patients the positive-feedback loop may contribute to the increased expression of gastrin mRNA within the tumour [25], and to the sustained increase in circulating gastrins [8,26,27]. The significance of this observation is that Ggly promotes the growth of various gastrointestinal cell lines [28–30], and acts as a co-carcinogen in vivo. Thus Ggly-treated rats have increased numbers of colorectal tumour precursors after treatment with azoxymethane [3], and polyp numbers were increased in $APC^{min/+}$ mice overexpressing Ggly [31].

Hypergastrinemia is a well known consequence of the increased use of proton pump inhibitors (PPI) to treat both acid secretory disorders and *Helicobacter pylori* infections. Although increased circulating Gamide causes gastric ECL hyperplasia in rats and synergises with *H. pylori* infection in mouse models of gastric adenocarcinoma, and both effects are mediated via the CCK2R, the general consensus is that Gamide does not contribute to the development of gastric neuroendocrine tumours or adenocarcinomas in humans [7]. Interestingly, therapy for 5 years or longer with high, but not low, dose PPIs is strongly correlated with an increased incidence of CRC [32]. The CCK2R, although not often expressed on CRC tumour cells [33], is found on polymorphs within the tumour stroma [34]. The data presented herein are consistent with the suggestion that PPI-induced hypergastrinemia may accelerate the development of CRC indirectly by stimulating the expression of non-amidated gastrins by such CCK2R-expressing polymorphs. Thus the regulation of the gastrin positive-feedback loop by hypergastrinemia and its role in CRC development clearly warrants further investigation.

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